For the reasons given below, Applicants respectfully traverse the requirement for restriction between Groups I, III, IV, V and VI, and request that the claims of these Groups be examined together inasmuch as they do possess a common inventive feature, namely the above-mentioned heptasaccharide.

Restriction among Groups I, III, IV, V and VI was required because the inventive feature of the heptasaccharide of claim 1 was deemed not novel based on its disclosure in the 2002 Young et al. publication. However, this publication in fact is not prior art to this Application.

The Young et al. publication states on its face that the first publication date was August 16, 2002. The current application, however, claims the priority of US provisional application 60/399,735 filed August 1, 2002 by five inventors, all of whom are named as authors in the publication. A copy of said application is enclosed for the examiner's convenience. That provisional application discloses the claimed heptasaccharide (see, e.g. the Abstract on page 29) and its identification, to the same extent as the Young et al. publication.

Inasmuch as this application is entitled to the priority of US provisional application 60/399,735, the Young et al. publication does not anticipate claim 1, and the commonness of inventive features among all claims, with the exception of claim 6, exists.

Applicants therefore respectfully request withdrawal of the requirement for restriction among Groups I, III, IV, V and VI.

For the same reasons, Applicants respectfully request withdrawal of the holding that claims 1-5 and 7-9 define different species under PCT Rule 13.1 and request that these claims be examined to their full scope.

CONCLUSION

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned.

Respectfully submitted,

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THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/399,735*
FILING DATE: *August 01, 2002*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

G. W. MONTGOMERY
Certifying Officer

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PROVISIONAL APPLICATION COVER SHEET

Approved

This is equest for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2)

Docket Number	11421-1	Type a Plus Sign (+) inside this Box -->	+

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TITLE OF THE INVENTION (280 CHARACTERS MAX.)

Structure of the N-linked Glycan Present on Multiple Glycoproteins the Gram-negative Bacterium, Campylobacter jejuni

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ENCLOSED APPLICATION PARTS (*Check all that Apply*)

X	*Specification*	*Number of pages*	29		*Small Entity Statement*	
X	*Drawing(s)*	*Number of Sheets*	8		*Other (specify)*	

METHOD OF PAYMENT (*Check One*)

	A check or money order is enclosed to cover the Provisional filing fees	*Provisional Filing Fee Amount ($)*	$ 160.00
X	*The Commissioner is hereby authorized to charge filing fees and any deficiency in the filing fees and credit to our Mastercard Account Form PTO-2038 is attached*		
	We hereby authorize you to deduct any deficiency of the fee stated above or credit to this amount to our Deposit Account 14-0429.		

The invention was made by an agency of the United States Government or under contract with an agency of the United States Government. X No

Date: 1 August 2002

Respectfully submitted,

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Enclosures
:ech

X Additional inventors are being named a separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

[redacted]

Docket Number	11421-1	Type a Plus Sign (+) inside this Box -->	+

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Structure of the N-linked Glycan Present on Multiple Glycoproteins in the Gram-negative Bacterium, *Campylobacter jejuni*

BACKGROUND OF THE INVENTION

Glycosylation of proteins was once considered to be specifically a eukaryotic phenomenon, but it is now clear that it is widespread in both the Archaea and Eubacteria domains (1;2). Glycosidic linkages of both the N- and O- types have been identified in a diverse group of prokaryotic organisms with a preponderance of N-linked sugars apparent in the Archaea while linkage units of the O-type predominate in glycoproteins identified thus far in the Eubacteria (1;2). In addition, bacterial N- and O-linkages are formed with a wider range of sugars than those observed in eukaryotic glycoproteins.

Recently a gene locus was identified in the enteric pathogen *Campylobacter jejuni*, which appears to be involved in the glycosylation of multiple proteins, and which provided the first evidence of a pathway for wide-spread protein glycosylation in a gram-negative bacterium (3). Mutagenesis of genes within this locus, termed *pgl* (for protein glycosylation), resulted in loss of immunogenicity in multiple proteins. The glycan moieties of these proteins were also shown to be recognized by antisera from experimentally infected human volunteers (3). Removal of the glycan moieties by *pgl* mutation resulted in decreased adherence and invasion *in vitro* and loss of mouse colonization *in vivo* (4), suggesting that protein glycosylation influences the virulence properties of this organism. A similar genetic locus has recently been described for pilin O-glycosylation in *Neisseria meningitidis* (5;6).

This glycosylation pathway appears to be distinct from that involved in the glycosylation of flagellin observed for several bacterial species (7-10). Thibault *et al.* (11) recently demonstrated that the glycosyl moiety on the flagellin of *C. jejuni* consists of variants of an unusual nine-carbon sugar, pseudaminic acid, O-linked to Ser or Thr. This sugar may be considered a highly modified sialic acid (12).

Recently, we have identified and characterized post-translational modifications of proteins in *C. jejuni* strain NCTC 11168, the strain for which the whole genome sequence has been described by Parkhill *et al.* (13). Among the proteins giving rise to multiple spots on 2D gels was PEB3, or Cj0289c, a major antigenic protein of *C. jejuni* first described by Pei *et al.* (14). When purified and analysed by 1D SDS-PAGE, it revealed two bands with a mass difference of ~1500 Da, both of which had N-terminal sequences corresponding to authentic PEB3. Concurrent with our observations on PEB3, Linton *et al.* (15) identified two putative glycoproteins from *C.jejuni* by use of the GalNAc-specific lectin, soybean agglutinin, one of which was PEB3, and the other a putative periplasmic protein Cj1670c, which they named CgpA. The authors also observed a number of other putative glycoproteins, based upon their ability to bind to the lectin, but these were not identified. Furthermore, protein binding to the lectin was also affected by mutagenesis of genes in the *pgl* locus.

SUMMARY OF THE INVENTION

According to the invention, we have analysed the *C.jejuni* NCTC 11168 glycoprotein complement and have identified individual glycoproteins and structurally

characterized the N-linked glycan moiety, which is common to all these glycoproteins, by mass spectrometry and NMR spectroscopy.

In addition, we have shown that mutation of a gene *pglB*, whose homology to the STT3 subunit of the N-linked oligosaccharyltransferase of *Saccharomyces cerevisae* suggested a role in glycoprotein biosynthesis (3;16), specifically affects the glycosylation of the identified glycoproteins.

BRIEF DECRIPTION OF THE DRAWING

FIG. 1. Purification and analysis of PEB3. a) Cation-exchange chromatography of a glycine extract on MonoS column. PEB3 containing fractions were identified by N-terminal sequencing of SDS-PAGE bands, pooled as indicated, dialyzed and freeze-dried. b) Re-fractionation of pooled material on MonoS column using a shallower gradient of 0-0.2 M NaCl. The inset shows SDS-PAGE analysis of fractions 30 and 31, which were identified as PEB3 containing fractions by N-terminal sequencing of SDS-PAGE bands.

FIG. 2. ESI-MS analysis of fractions from cation exchange chromatography purification of PEB3. a) ESI-MS of the fraction 31 of Figure 1, after dialysis and the reconstructed molecular mass profile obtained from this spectrum b). The peaks at 25,454 and 28,376 Da were identified as PEB3 and PEB4, respectively. Further analysis was required to identify the third peak at 26,861 Da as glycosylated PEB3. c) ESI-MS of PEB3 similarly purified from the *pglB* mutant, and the reconstructed mass profile (d), showing the lack of the glycosylated form of PEB3.

FIG. 3. **MS/MS analysis of the PEB3 tryptic glycopeptide.** a) Product ion spectrum of the doubly protonated glycopeptide ion at m/z 1057.9. The fragment ions originating from the sequential loss of oligosaccharide residues are indicated in the spectrum. The peptide sequence is shown in the inset. b) Second generation product ion spectrum of the glycopeptide fragment ion at m/z 937.4. The glycopeptide was fragmented by front end collision induced dissociation (orifice voltage = 100 V) as it entered the mass spectrometer. The observation of the b_3+228 fragment ion at m/z 605.3 confirmed that the oligosaccharide is N-linked.

FIG. 4. **2D gels of the SBA affinity chromatography product.** The proteins were separated on 2D-PAGE in two pH ranges, pH 4-7 (a) and pH 6-11 (b), and then silver-stained. The identities of the spots, shown by their Cj numbering, were determined by mass spectrometry of their tryptic digests. A full list of the identified proteins is given in Table I.

FIG. 5. **Purification of glycopeptides from a pronase digest of the SBA affinity chromatography product.** a) Size exclusion chromatography on BioGel P4 200 mesh of the pronase digest. b) Re-fractionation of pooled material from P4 on BioGel P2 fine grade. In both fractionations, glycoprotein-containing fractions were identified by MS and pooled as indicated by the bars. c) ESI-MS spectrum of fraction 10 from B above. The doubly protonated ion (MH_2^{2+}) at m/z 770.5 corresponds to the heptasaccharide linked to Asn. A number of larger ions are also observed and are due to the addition of a second amino acid residue. The amino acid compositions of the major glycan-containing ions are indicated on the spectrum.

FIG. 6. Structure and ^1H spectra of the glycopeptide. (a) Deduced structure of the glycopeptide. (b) Spectrum after final purification on a P2 column (D_2O, 35°C, 1 mM deuterated EDTA). The anomeric sugar resonances in the region 4.4 to 5.4 ppm are labeled.

FIG. 7. 1D selective NMR experiments with the glycopeptide. (a) 1D TOCSY(g1f2, 15 Hz, 144 ms), (b) 1D TOCSY(e1f1, 25 Hz, 144 ms), (c) 1D TOCSY(d1, 20 Hz, 144 ms), (d) 1D TOCSY(b6, 50 Hz, 66 ms) where * is a peptide resonance, (e) 1D TOCSY(b1c1, 40 Hz, 151 ms), (f) 1D TOCSY(a1, 25 Hz, 144 ms), (g) 1D NOESY(g1f2, 15 Hz, 400 ms), (h) 1D NOESY(f1, 10 Hz, 400 ms), (i) 1D NOESY(e1, 10 Hz, 400 ms), (j) 1D NOESY(d1, 20 Hz, 400 ms), (k) 1D NOESY(b1c1, 40 Hz, 400 ms), (l) 1D NOESY(a1, 40 Hz, 400 ms).

FIG. 8. The *pgl* locus and characterization of the *pglB* mutant. a) Gene schematic of the general protein glycosylation locus of *C. jejuni* NCTC 11168. Genes which have homologues to genes in the *pgl* locus of *Neisseria spp.* are shown by grey arrows. The mutation in *pglB* shown below the locus was constructed using pEAp26. b-e) Two dimensional gel analysis of *C. jejuni* wildtype and isogenic *pglB* mutant. b) and d) Colloidal coomassie stain of 2D gels before immunoblotting of wildtype and mutant, respectively. c) and e) Immunodetection of proteins by HS:2 serotyping sera of wildtype and mutant, respectively. The arrows indicate the proteins which showed differences in either gel migration and/or immunoreactivity which were excised for identification by mass fingerprinting. The

identities of the spots are shown by their Cj numbering. The masses of the molecular weight protein markers in kDa are shown at the left.

DETAILED DESCRIPTION OF THE INVENTION

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids - *C.jejuni* NCTC 11168 was routinely grown on Mueller Hinton agar under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2) at 37°C. *E. coli* DH10B (Invitrogen) was used as the host strain for cloning experiments and clones were grown on Luria S-gal agar (Sigma) or MH agar at 37°C. When appropriate, antibiotics were added to the following final concentrations: kanamycin 30 µg/mL and ampicillin 150 µg/mL. Plasmid pPCR-Script Amp (Stratagene) was used as the cloning vector.

Preparation of glycoprotein extracts – Cells from two plates of overnight growth were re-suspended in 10 mL Mueller Hinton broth and used to inoculate 1 litre of MH culture medium. Cultures were grown under microaerophilic conditions at 37°C for 24 h with shaking at 150 rpm. Bacterial cells from 12 litres of culture media were harvested by centrifugation at 10,000 x *g* for 15 min and immediately frozen at -75°C. Frozen cell pellets were thawed on ice in 0.2 M glycine HCl buffer, pH 2.2 (17), and extracted for 15 min with gentle stirring. Extracts were clarified by centrifugation at 10,000 x *g* for 15 min, dialyzed against pure water (Milli-Q system, Millipore Corporation) and freeze-dried.

Purification and analysis of PEB3 - The PEB3 protein was purified to homogeneity by cation exchange chromatography of the glycine extract as previously described (14). A Pharmacia MonoS HR 5/5 column was used on an

ÄKTA Explorer LC system (Amersham Biosciences). The column eluate was monitored for UV absorbance at 280 nm and fractions were examined by SDS-PAGE analysis (18) in Mini Protean II slab gels (BioRad Laboratories). N-terminal sequencing of individual proteins was performed on a model 491 Procise protein sequencing system (Applied Biosystems Inc.), following transfer from SDS gels to ProBlot™ PVDF membrane (Applied Biosystems Inc.) as described by LeGendre *et al.* (19).

The protein molecular weight profiles of selected fractions were determined by electrospray ionization mass spectrometry using an Applied Biosystems/Sciex Q-Star hybrid quadrupole time-of-flight mass spectrometer. The fractions were first dialyzed extensively to remove salts, and adjusted to 30% methanol, 0.2% formic acid. The solution was infused at a flow rate of 1 µL/min and spectra were acquired over the range m/z 600>2000.

Analysis of tryptic peptides - Selected fractions were digested overnight at 37°C with modified trypsin (Promega) in 50 mM ammonium bicarbonate and analyzed by capillary LC-tandem mass spectrometry using a capillary HPLC system (CapLC, Waters) coupled with a Q-TOF2 hybrid quadrupole time-of-flight mass spectrometer (Micromass). Approximately 250 ng of each digest was injected on a 0.3 x 150 mm PepMap C_{18} capillary LC column (Dionex/LC-Packings) and resolved by gradient elution (5-90% acetonitrile, 0.2% formic acid in 45 minutes). The mass spectrometer was set to operate in automatic MS/MS acquisition mode and spectra were acquired on doubly, triply and quadruply charged ions.

Larger scale separation of the tryptic digest was carried out on a 4.6 mm x 250 mm Jupiter C_{18} LC column (Phenomenex Inc.). The fraction containing the glycopeptide was then infused at a flow rate of 1 µL/min into the microelectrospray

interface of the Q-TOF2 mass spectrometer. Fragmentation of the glycopeptide prior to MS/MS analysis was achieved by front-end collision-induced dissociation (the orifice voltage was increased to 100 V from the normal 40 V). The MS/MS collision offset for the singly charged fragment ions produced in this manner was 20-25 V (lab frame of reference). For β-elimination experiments by the method of Rademaker *et al.* (20), approximately half of the glycopeptide-containing fraction was evaporated to dryness and dissolved in 25% aqueous ammonium hydroxide. The solution was left at room temperature overnight, evaporated to dryness for a second time and re-dissolved in water. The solution was then examined by infusion-MS as described above.

Purification and analysis of total glycoproteins - The glycoproteins from the glycine extracts were isolated by affinity chromatography on SBA[1] lectin/agarose (Sigma-Aldrich Ltd.). The freeze-dried glycine extract was re-dissolved in PBS (100 mM NaCl, 50 mM sodium phosphate pH 7.5) and passed through an SBA/agarose column previously equilibrated in PBS. The column was washed with 10 column volumes of PBS and bound glycoprotein was eluted with 0.1 M GalNAc in PBS. Glycoprotein-containing fractions were pooled, dialyzed against Milli-Q water and freeze-dried.

The glycoproteins were separated by SDS-PAGE on 12.5% homogeneous polyacrylamide gels (18). 2D-PAGE was performed using pre-cast IEF strips containing immobilized linear pH gradients of either pH 3-10, pH 4-7 (BioRad Laboratories) or pH 6-11 (Amersham Biosciences). Proteins were solubilized in sample buffer according to the manufacturer's instructions and resolved by isoelectric focussing on the precast IEF strips followed by SDS-PAGE on homogenous 12.5% slab gels, 20 x 20 cm, for the second dimension. Gels were

stained with Bio-Safe colloidal G-250 Coomassie blue stain (BioRad Laboratories) or silver stained (20). For subsequent lectin probing, the gels were electroblotted onto PVDF membrane at 50 V for 1 h in 10 mM 3-(cyclohexylamino)-1-propane sulphonic acid buffer, pH 11, containing 10% methanol. The membrane was washed in Milli-Q water and blocked in Tris-buffered saline (100 mM NaCl, 50mM Tris pH 7.5) with 0.05% Tween 20 and 2% blocking buffer (Roche Molecular Biochemicals) for 2h at room temperature. Following blockage, blots were further incubated with SBA-alkaline phosphatase conjugate (EY Laboratories Inc.) at a concentration of 10 µg/mL in the above blocking solution for 1h at room temperature. Blots were washed three times in Tris-saline with 0.05% Tween 20 and developed using nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.1 M NaCl, 0.1 M Tris pH 9.5 with 50 mM $MgCl_2$.

MS analyses of 2D-gel spots - The protein spots were excised and destained with a 1:1 ratio of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (21). The gel spots were washed extensively with deionized water, shrunk with acetonitrile, re-swollen with 50 mM ammonium bicarbonate containing Promega modified trypsin (10 ng/µL) and sufficient 50 mM ammonium bicarbonate was added to cover the gel pieces (typically 30 µL). The tubes were sealed and incubated overnight at 37°C. The digest solutions were removed and the gel pieces were extracted with 50 µL of 5 % acetic acid and then with 50 µL of 5% acetic acid in 50% aqueous methanol. The extracts were pooled with the digest solutions and concentrated to approximately 10 µL.

The peptide extracts from the intense protein spots were analyzed by MALDI-TOFMS using a M@LDI-LR mass spectrometer (Micromass). Approximately 0.5 µL of the MALDI matrix solution (10 mg/mL α-cyano-4-hydroxy-cinnamic acid in 50%

acetonitrile, 0.2% TFA) was deposited on the target plate and allowed to dry. The peptide extracts were desalted using C_{18} ZipTips™ (Millipore) and were deposited directly on the matrix spots. Acquisition of the MALDI-TOFMS spectra was carried out automatically. The spectra were calibrated externally using peptide standards and internally with trypsin autolysis peptides. Database searching was carried out in batch mode using Mascot Daemon™ (Matrix Science) and against the *C. jejuni* NCTC11168 genome sequence database.

The extracts from the fainter protein spots were analyzed by nanoLC-MS/MS using the Q-TOF2 mass spectrometer. The entire samples were injected onto a 0.3 x 5 mm C_{18} micro pre-column cartridge (Dionex/LC-Packings). The peptides were retained while the sample solution was washed to waste. The trap was then brought online with a 75 μm x 150 mm C_{18} Nano-Series column (Dionex/LC-Packings) and the peptides were separated with a gradient supplied by the CapLC pump (15-75% acetonitrile, 0.2 % formic acid in 30 minutes, approximately 300 nL/min flow rate). The mass spectrometer was set to acquire MS/MS spectra in automated mode as described above. Database searching was carried out as described for the MALDI-TOFMS analyses.

Glycopeptide preparation - Freeze-dried total glycoprotein (5 mg) was dissolved in 250 μL of 100 mM Tris pH 8.0 containing 2 mM $CaCl_2$, and digested with pronase as previously described (22). The digest was microfuged at 10,000 x *g* for 15 min and the supernatant was applied to a column (1 x 120 cm) of BioGel P4, 200 mesh (BioRad Laboratories) The column was run in water and the column eluate was monitored by refractive index. Fractions were screened by ESIMS and precursor ion scanning mass spectrometry (precursors of the HexNAc oxonium ion at m/z 204) on an API 3000 triple quadrupole mass spectrometer (Applied

Biosystems/Sciex). Fractions giving the HexNAc ion signature were pooled and freeze-dried. The glycopeptides were further purified on a 1 x 120 cm column of BioGel P2 fine grade, the fractions being monitored and screened as described above.

NMR spectroscopy - All spectra were acquired using a Varian Inova 600 MHz spectrometer using the standard Varian software. A gradient 4 mm indirect detection high-resolution magic angle spinning nano-NMR probe (Varian) with a broadband decoupling coil was used. The sample was spun at 3 KHz with dry nitrogen as the drive and bearing gas. The spin rate was not computer controlled but remained constant to within 10 Hz of the set value. Samples in 40 µL D_2O solution were recorded at 25°C and at 35°C to produce sharper peaks. The pH was unknown due to the small volume. Deuterated EDTA (CDN Isotopes Inc.) was added to chelate metal ions and provide sharper peaks for bacillosamine and amino acids. Although the glycopeptide isolate from a P4 column contained some amino acid and sugar impurities, spectra were of sufficient quality to allow complete resonance assignments of the glycopeptide in the presence of 15 mM of deuterated EDTA. Much of the NMR structural work proceeded with this sample because of the risk of loosing the bulk of the isolated glycopeptide by further purification. The derived structure was confirmed by additional NMR experiments on glycopeptide that had been purified using a P2 column, lyophilized, and dissolved in 40 µL D_2O with 1 mM deuterated EDTA. The experiments were performed with suppression of the HDO signal at 4.78 ppm (25°C) and 4.67 ppm (35°C). Acquisition and processing of two-dimensional experiments (COSY, TOCSY, NOESY, HMQC, HMBC) were performed as described previously (23). The ^1H reference was set by external acetone at 2.23 ppm. The ^{13}C reference was set with the methyl resonance

of external acetone at 31.07 ppm. The ^{1}H and ^{13}C chemical shifts in Table 1 were measured from the proton spectra and from C-H cross peaks in the HMQC and HMBC spectra. 1D TOCSY experiments with various spin-lock times from 30-151 ms and 1D NOESY with mixing times from 400-800 ms were performed as described previously (23;24). Selective experiments were described as 1D EXP(selected spins, selective excitation bandwidth, mixing time) where EXP is TOCSY or NOESY.

The use of magic angle spinning (MAS) for liquid state samples in the presence of both RF and magnetic-field homogeneities has been shown to influence significantly the performance of mixing sequences in TOCSY experiments and can degrade performance (25;26). Using adiabatic (WURST) mixing sequences can eliminate such effects (25;27). The standard 2D TOCSY and 1D TOCSY sequences were modified so that the MLEV-17 or DIPSI-2 mixing sequence was replaced with the adiabatic WURST-2 pulses. The adiabatic (WURST-2) mixing had a single adiabatic inversion pulse length of T_p= 1/MAS spin rate, a modulation depth of 8 and an adiabicity of 2. Typically, for the WURST-2 pulse, the sweep bandwidth was 24 kHz, T_p = 0.333 ms (at a MAS spin rate of 3000 +/- 10 Hz), B_1 (max)= 8.51 kHz, B_1 (RMS) = 4.77 kHz.

GC-MS analysis - The enantiomeric configurations of the Glc and GalNAc components of the P2 product were assigned by characterization of the but-2-yl glycosides in gas liquid chromatography – mass spectrometry (28). The derivatives were analyzed using a Hewlett-Packard chromatograph equipped with a 30m DB-17 capillary column (180°C to 260°C at 3.5°C/min), and spectra in the electron impact mode were obtained with a Varian Saturn II mass spectrometer.

Construction and characterization of pglB *mutant* - For construction of the *pglB* mutant, genes Cj1121c to Cj1126c were PCR amplified from *C. jejuni* NCTC 11168 using the primers: Cj1121cF (5'-ACTCACTATTGCCATTAAGATAAGC-3') and Cj1126cR (5'-AAAACCCTTATTTAGTTTTGTTTGC-3'). The PCR product was polished with *Pfu* polymerase and then ligated into pPCR-Script Amp (Stratagene) according to the manufacturer's instructions. The ligation mixture was electroporated into electrocompetent *E. coli* DH10B and selected for on LB S-gal agar (Sigma-Aldrich) with ampicillin. A blunt-ended kanamycin resistance cassette from pILL600 (29) was inserted into the filled-in *Xba*I restriction site of *pglB*, generating pEAp26. The orientation of the cassette was determined by sequencing with the ckanB primer (5'-CCTGGGTTTCAAGCATTAG-3'). DNA was sequenced using terminator chemistry and AmpliTaq cycle sequencing kits (Applied Biosystems) and analysed on an Applied Biosystems 373 DNA sequencer. The mutated plasmid DNA was used for electroporation into *C. jejuni* NCTC 11168 (30) and the kanamycin-resistant transformants were characterized by PCR to confirm that the incoming plasmid DNA had integrated by a double cross-over event.

Proteins were extracted from *C. jejuni* whole cells using 0.2 M glycine at pH 2.2 (17) and dialysed against water. Samples were analyzed by 2D-PAGE using 11 cm pH 3-10 ReadyStrip IPG strips (BioRad Laboratories) and pre-cast 12 x 8 cm 8-16% gradient Criterion slab gels (BioRad Laboratories). Gels were stained with colloidal coomassie blue, photographed, and then partially destained by washing in water. Proteins were transferred for 1 h at 207 mA onto PVDF membranes using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad). After blocking overnight, membranes were probed with a 1:500 dilution of HS:2 serotyping serum followed by

a 1:5000 dilution of goat anti-rabbit antiserum (Sigma-Aldrich), and developed with NBT/BCIP (Roche Molecular Biochemicals).

RESULTS

Purification and characterization of PEB3 - PEB3 protein (Cj0289c) was identified in 2D gels of a glycine extract by peptide mass fingerprinting, as a component of a group of spots focussing within a range of pH 9-10 (results not shown). PEB3 was purified from the extract by cation exchange chromatography, and re-fractionated on the same column, using a shallower NaCl gradient, resulted in the PEB3 appearing in three fractions (Fig.1). SDS-PAGE analysis showed two bands, whose N-terminal sequences were determined following their transfer to a PVDF membrane. Ten cycles of sequencing identified the lower mass species as PEB3 while the higher mass, more abundant component, was also PEB3 with a minor sequence corresponding to PEB4 (Cj0596).

The mass spectrum and the reconstructed molecular mass profile for fraction # 31 are presented in Figure 2a,b. Three peaks were observed in the reconstructed mass profile. The peaks at 25,454 Da and 28,376 Da correspond well with the expected molecular masses of PEB3 (25,453 Da, Cj0289c) and PEB4 (28,377 Da, Cj0596) respectively, without signal peptides. To identify the protein of mass 26,861 Da, CapLC-MS/MS analysis was carried out on the tryptic digest of this fraction. All but one of the peptides identified could be assigned to PEB3 or PEB4, in accord with the N-terminal sequence data. MS/MS analysis of the unidentified ion (Fig. 3a) clearly identifies it as a glycopeptide. A fragmentation series composed of sequential losses of HexNAc (203 Da) and a single Hex (162 Da) can be observed in this spectrum. The tryptic peptide was identified as ^{68}DFNVSK^{73} from PEB3. The

residue mass of the oligosaccharide portion of this glycopeptide is 1406 Da, which corresponds well with the difference in the molecular weights of PEB3 and the unknown protein peak observed in Figure 2b. Therefore, it appeared that approximately 50% of the PEB3 protein in this fraction was modified with a single oligosaccharide composed 5 HexNAcs, 1 Hex and an unusual sugar with a residue mass of 228 Da. Moreover, the MS/MS spectrum indicated that the oligosaccharide was linked to the peptide via the 228 Da sugar moiety.

Characterization of the glycopeptide linkage - The PEB3 tryptic peptide to which the oligosaccharide is attached contains sites for both N- and O-linkage, ie Asn and Ser. Therefore, it was necessary to carry out further experiments to determine the nature of this linkage. Previously, we have used β-elimination to remove O-linked carbohydrates from the flagellin of *C. jejuni* 81-176 (11). However, this procedure failed to remove the oligosaccharide in this instance. This was our first indication that the oligosaccharide is N-linked. This was confirmed by MS/MS analysis of the singly protonated fragment ion at m/z 937.0 produced by front-end collision-induced dissociation of the intact glycopeptide (Fig. 3b). This ion is composed of the tryptic peptide plus the unusual 228 Da sugar only. An ion was observed at m/z 605.1 which could only be assigned to the b_3 fragment ion plus the 228 Da sugar moiety. No fragment ions were observed to suggest that the carbohydrate is linked to the serine residue. All of this evidence strongly suggests that the oligosaccharide is linked to the peptide at Asn70. Interestingly, this peptide contains the eukaryotic N-linkage consensus sequon, Asn-Xaa-Ser.

Isolation and identification of glycoproteins - Putative glycoproteins were purified by SBA affinity chromatography from the glycine extracts of 40 g wet weight of cells. The yield of putative glycoproteins was 5 mg as estimated by UV

absorbance at 280 nm. The GalNAc eluant was subjected to 1D- and 2D-PAGE (Fig. 4) and to ensure that the proteins purified in this manner possessed lectin binding properties, rather than non-specific binding characteristics, western blotting with an SBA/alkaline phosphatase conjugate was also carried out. Approximately 13 protein species were visualized following 1D SDS-PAGE but this number increased substantially when the product was analyzed by 2D-PAGE. The proteins in individual bands from 1D SDS-PAGE and spots from 2D-PAGE were identified by mass fingerprinting and database searching (Table I). Among the identified proteins are PEB3 (Cj0829c) and CgpA (Cj1670c) previously identified by Linton *et al.* (15). The vertical pattern of spots with identical pIs displayed by Cj1670c, and other proteins, likely indicates varying degrees of glycosylation since examination of their predicted amino acid sequences, derived from the whole genome sequence of *C.jejuni* NCTC 11168 (13), revealed the presence of multiple potential N-linked glycosylation sites containing the sequon Asn-Xaa-Ser/Thr (Table I). In fact, MS/MS analysis of the Cj1670c-containing in-gel digest extracts indicated that 3 of its 6 N-linkage sites are occupied to varying extents (three Cj1670c glycopeptides were detected by capLC-MS/MS: ^{7}TDQ<u>N</u>ITLVAPPEFQKEEVK^{25}, ^{77}VLDVSVTIPEK<u>N</u>SSK^{91} and ^{92}QES<u>N</u>STANVEIPLQVAK^{108}. A single glycopeptide was also observed for Cj0114 (^{71}LSQVEENNQNIEN<u>N</u>FTSEIQK^{91}) and for Cj0200c (^{1}DSLKLEGTIAQIYDN<u>N</u>K^{17}). Furthermore, the mass and composition of the glycan component of all these glycopeptides appears to be identical to that observed for PEB3.

However, certain proteins identified from the 2D-PAGE, notably Cj0147c, Cj0169, Cj0332c, Cj0334, Cj0638c, Cj1181c and Cj1534c do not contain any of these specific sequons in their amino acid sequences. These proteins either are

non-covalently associated with SBA-binding proteins or bind non-specifically to the column. This conclusion is supported by the failure of these protein spots to react with the SBA/alkaline phosphatase conjugate following 2D PAGE and electroblotting (Table I).

Preparation of glycopeptides – The mixed glycoprotein sample was subjected to two rounds of pronase digestion and the products were separated by gel filtration on BioGel P4 (Fig. 5a). The carbohydrate-containing fractions were located by mass spectrometry and, after NMR studies (see below), the sample was re-purified on BioGel P2 (Fig. 5b). The final purified sample was composed mainly of the oligosaccharide linked to a single Asn (Fig. 5c). There was no evidence of any variation in the glycan component. The yield of glycopeptides was estimated at around 200 μg.

NMR spectroscopy - With the use of selective methods, it was possible to work with the impure P4 sample. The complete resonance assignment of the sugar moiety was done with this sample for fear of loss of glycopeptide upon further purification. After a second purification using a P2 column, 25% of the glycopeptide was lost as judged by measurement of the S/N ratio for the P4 and P2 purified samples. An HMQC experiment was rerun on the P2 purified sample to confirm the assignments obtained using the P4 purified sample. From mass spectrometry results, the glycopeptide was composed of 5 HexNAc residues, a Hex residue, and an unknown sugar with a mass of 228 Da. The absolute configuration of the HexNAc and Hex residues was determined to be D by chemical analysis. Analysis of the ^{1}H and ^{13}C NMR data indicated the presence of 8 anomeric protons labeled in alphabetical order (Fig. 6). A series of 1D TOCSY for the anomeric resonances was done for proton assignments (Fig. 7). Different mixing times were used to assign

the spins within each residue. HMQC and HMBC were then used to assign the ^{13}C resonances. The NMR assignments are given in Table II. 1D NOESY experiments (Fig. 7) were used to obtain the sequence as shown (Fig. 6).

Residue g was assigned to β-D-Glc*p*. For the 1D TOCSY of g1 with a mixing time of 144 ms (Fig. 7a), all spins up to the H6 and H6' resonances were detected, indicative of the large $J_{H,H}$ couplings typical of β-glucopyranose. Resonance g1 also overlapped with f2, allowing detection of the f1 to f4 resonances. Residue g was terminal due to similar ^{13}C and ^{1}H chemical shifts for C2 to C6 with those of β-D-glucopyranose (31).

Five residues (a, c, d, e, and f) were identified as α-D-Gal*p*NAc. A value of $J_{1,2}$ of 3.6 +/- 0.2 Hz, the strong H1-H2 NOE (Fig. 7), and $J_{H,H}$ coupling pattern which included a small coupling to H4 (Fig. 7) showed that these units had the α-D-galactopyranosyl configuration. Although, the e1 and f1 anomerics resonances could be selectively excited with a narrow bandwidth of 10 Hz, the spectra for simultaneous excitation of e1 and f1 are shown in Figure 7b. Resonances f2 to f4 were also detected for the 1D TOCSY of g1f2 (Fig. 7a). A chemical shift for C2 near 51 ppm was indicative of an acetamido group. The (C2, H4) HMBC correlations were used to assign the C2 resonances of the five GalNAc residues. The 1D TOCSY-NOESY(H1, H4) was used to detect the NOE between H4 and H5 and thus assign the H5 resonances (23). The 1D-TOCSY experiments on the H5 resonances were then used to detect the H6s resonances (not shown). Integration of the P2 purified sample (Fig. 6b) indicated 7 NAc groups, five of those corresponding to the five GalNAc residues. Comparison of the ^{13}C chemical shifts of the GalNAc units with those of α-D-Gal*p*NAc indicated that residues a, c, e, f were linked at O-4 due to downfield shifts for C4 (32). Residue f had a branch point at O-3 as established

by a downfield shift for C3. Residue d was terminal due to similar ^{13}C chemical shifts for C2 to C6 with those of α-D-Gal*p*NAc (32).

Residue b was assigned to ß-D-bacillosamine (2,4-diacetamido-2,4,6-trideoxy-ß-D-glucopyranose). For residue b and c, the anomeric resonances partially overlapped. For the 1D TOCSY of the b1 and c1 resonances with a mixing time of 144 ms (Fig. 7e), broad resonances could be identified up to a CH_3 resonance (b6) at 1.14 ppm. For the 1D TOCSY for b6 at 1.14 ppm, with a mixing time of 66 ms (Fig. 7d), peaks up to b4 were observed. A series of 1D TOCSY with different mixing time from 30 ms to 144 ms were done to assign the peaks. For residue b, the broad peaks do not provide the coupling constants. However, for the 1D TOCSY of b1 with a mixing time of 144 ms, resonances up to H6 could be observed, similar to the 1D TOCSY for H1 of β-Glc*p* (residue g). Hence, such efficient transfer was indicative of large coupling constants typical of a ß-glucopyranosyl configuration. The b1-b3 and b1-b5 NOEs observed in Fig. 7k were also typical of the ß anomeric configuration. Chemical shifts for C2 and C4 at 55 and 58 ppm were indicative of acetamido groups, in accord with the presence 7 NAc groups in the structure (Fig. 6a). The chemical shift for C1 at 79 ppm and H1 at 5.1 ppm indicated an N-linked anomeric similar to that found for ß-GlcNAc-Asn (33). Comparison of the chemical shifts of residue b with those of 2,4-diamino-2,4,6-trideoxy-β-D-glucopyranose found in other structures indicated that Bac was linked at O-3, the only possible glycosidation site (34-36).

The absolute configuration of residue b was obtained by NOEs as previously described for another structure containing bacillosamine (34). A strong NOE observed between the CH_3 resonance at 1.14 ppm (b6) and the NAc at 1.95 ppm was due to the close proximity of the CH_3 group at C6 to the NAC-CH_3 group at C4.

Since, the NAc resonance at 1.95 ppm was isolated from the other NAC resonances it could be selectively excited. A strong NOE was observed between this NAc resonance and the α-D-GalpNAc H1 resonance of residue a, due to the a(1-3)b linkage as shown below. This NOE can only occur if residue b has the D-configuration, where the NAc group at C4 of residue b is in close proximity (3 Å) to the anomeric proton of residue a. This NOE is not possible if residue b has the L-configuration, since the H1a / 4b-NAc interproton distances are greater than 5 Å.

The sequence was determined by 1D NOESY experiments (Fig. 7). The strong c1-f4 NOE and smaller c1-f6 and c1-f6' NOEs established the c(1-4)f linkage. The strong d1-c4 NOE and a smaller d1-c6' indicated the d(1-4)c sequence. The e1-a4, f1-e4 and g1-f3 established the e(1-4)a, f(1-4)e and g(1-3)f linkages. The a1-b3 NOE (Fig. 7l), established the a-b sequence. The structure is shown in Figure 6a. The sequence is in accord with the glycosidation shift observed for the linkage carbons (see above).

Characterization of a pglB *mutant* – A *pglB* mutant was constructed by cassette mutagenesis (Fig. 8). Glycine extracts of the mutant cells demonstrated dramatic changes in protein immunoreactivity by 2D-PAGE with western blotting with HS:2 serotyping sera (Fig. 8b,e). Several of the proteins which showed a change in the mobility and/or immunoreactivity on 2D gels were identified by mass fingerprint analysis (Table I). The protein identifications are in agreement with those identified by SBA lectin affinity chromatography, providing further evidence that the proteins reactive with the GalNAc lectin are glycosylated by the Pgl pathway. The full set of SBA-reactive proteins was not observed in this experiment since it was performed on whole glycine extracts and at different pH range, ie Figures 4 and 8 are not directly comparable.In addition, PEB3 was purified from the *pglB* mutant by

ion-exchange chromatography as described above, and analysis by mass spectrometry showed that the protein completely lacked the glycan (Fig. 2c,d).

To show that the *pglB* mutation only affected the glycosylation of the glycoproteins, analyses were performed of the lipooligosaccharide and capsular polysaccharide of the mutant. Deoxycholate-PAGE of proteinase K digests and mass spectrometry showed that the mass of the mutant LOS core was identical to that of the wildtype (results not shown). In addition, identical capsular repeats were visible in the extracts of the cells of the wildtype and the isogenic mutant on deoxycholate-PAGE. To further demonstrate that the capsule was unaltered, we examined the polysaccharide by HR-MAS NMR, which has been developed to examine capsular polysaccharide resonances on intact Campylobacter cells without the need for extensive growth and purification (St. Michael *et al.*, in preparation). The spectrum of the mutant was unchanged compared to that of the wildtype (results not shown).

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FOOTNOTES

[1] The abbreviations used are: Bac, bacillosamine, 2,4-diacetamido-2,4,6-trideoxy-D-glucopyranose; capLC-MS/MS, capillary liquid chromatography-tandem mass spectrometry; COSY, correlated spectroscopy; DIPSI-2, decoupling in the presence of scalar interactions; ESI-MS, electrospray ionization mass spectrometry; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple quantum correlation; HR-MAS, high-resolution magic angle spinning; LOS, lipooligosaccharides; MALDI-TOFMS, matrix assisted laser desorption time-of-flight mass spectrometry; MAS, magic angle spinning; MLEV-17, Malcolm Levitt's decoupling cycle; NOESY, nuclear Overhauser effect spectroscopy; SBA, soybean agglutinin; TOCSY, total correlation spectroscopy; WURST-2, wideband, uniform rate, and smooth truncation

TABLE I

Identification of proteins from 2-D gels

Cj Gene	Annotation	Number of sequons[a]	SBA staining[b]	*pglB* mutant[c]
Cj0114[d]	probable periplasmic protein	2S, 3T	+	+
Cj0143c	periplasmic solute binding protein for ABC transport system	1S, 1T	+	
Cj0147c	thioredoxin (TrxA)		-	
Cj0169	superoxide dismutase (Fe; SodB)		-	
Cj0175c[e]	putative iron uptake ABC transport system periplasmic iron binding protein	1S, 2T	+	+
Cj0200c[d]	probable periplasmic protein	1T	+	+
Cj0238	probable integral membrane protein	5S, 1T	-	
Cj0289c[d]	major antigenic peptide (PEB3)	2S	+	+
Cj0332c	nucleoside diphosphate kinase (Ndk)		-	
Cj0334	alkyl hydroperoxide reductase (AhpC)		-	
Cj0376	probable periplasmic protein	1S, 1T	+	
Cj0415[e]	putative oxidoreductase sub-unit	3T	+	
Cj0420	probable periplasmic protein	1T	±	+
Cj0493	translation elongation factor EF-G (FusA)	1T	±	
Cj0511	probable secreted proteinase	2S, 2T	+	+
Cj0638c	inorganic pyrophosphatase (Ppa)		-	
Cj0694	probable periplasmic protein	4S, 2T	+	
Cj0715	transthyretin-like periplasmic protein		-	+

Cj Gene	Annotation	Number of sequons[a]	SBA staining[b]	*pglB* mutant[c]
Cj0779	thioredoxin peroxidase (Tpx)	1S	+	
Cj0835c	aconitate hydratase (AcnB)	1S, 2T	+	
Cj0843c[e]	putative secreted transglycosylase	5S, 3T	+	
Cj0906c	probable periplasmic protein	2S, 2T	+	
Cj0944c	probable periplasmic protein	1S, 1T	+	
Cj0998c	probable periplasmic protein	1S, 1T	+	+
Cj1018c	branched-chain amino-acid ABC transport system periplasmic binding protein	1S, 2T		+
Cj1032	probable membrane fusion component of efflux system	2T	+	
Cj1181c	translation elongation factor EF-Ts (Tsf)		-	
Cj1214c	hypothetical protein	1S	±	
Cj1221	60 kD chaperonin (Cpn60; GroEL)	1S, 2T	+	
Cj1345c	probable periplasmic protein	5S, 2T	+	
Cj1380	probable periplasmic protein	2T	-	
Cj1444c[e]	putative capsule polysaccharide export system periplasmic protein (KpsD)	3S, 2T	+	
Cj1496c	probable periplasmic protein	1S, 1T	+	+
Cj1534c	probable bacterioferritin		-	
Cj1565c	paralysed flagellum protein (PflA)	5S, 2T	+	
Cj1643	putative periplasmic protein	3S, 1T		+
Cj1659	periplasmic protein (P19)		-	
Cj1670c[d]	probable periplasmic protein (CpgA)	4S, 2T	+	+

[a] S, Asn-Xaa-Ser sequons; T, Asn-Xaa-Thr sequons.

[b] Reactivity with SBA in Western blots of 2D gels.

[c] Proteins with changed spot position and/or immunoreactivity in 2D gels, in the *pglB* mutant. Cj1018c & Cj1643 were not isolated by SBA chromatography.

[d] Glycopeptides observed by CapLC-MS/MS.

[e] Identified from 1-D gel.

TABLE II

Chemical shifts (ppm) of the C. jejuni Asn-linked glycopeptide.

Measured at 600 MHz (^1H) in D_2O, 35°C with HOD at 4.67 ppm. External acetone methyl resonance at d_H 2.23 ppm and d_C 31.07 pppm. Error on d_C is ± 0.2 ppm and ± 0.02 ppm for d_H. Seven NAc resonances at d_H 2.07, 2.05, 2.04, 2.03, 2.02, 2.02 and 1.95 ppm. NAc-CH_3 d_C at 23.1-23.4 ppm. NAc-CO d_C at 175-176 ppm.

Residue	C1	C2	C3	C4	C5	C6	
	H1	H2	H3	H4	H5	H6	H6'
(a) a-GalNAc	98.0	50.7	68.0	77.6	72.7	62.2	
	5.21	4.21	3.83	4.02	3.83	3.93	3.90
(b) ß-Bac	79.0	54.5	76.3	58.1	75.0	17.6	
	5.11	3.91	3.91	3.80	3.59	1.14	
(c) a-GalNAc	98.3	51.6	67.9	77.5	71.8	60.9	
	5.10	4.26	4.14	4.10	4.46	3.71	3.62
(d) a-GalNAc	99.7	51.4	68.4	69.6	72.3	60.7	
	5.04	4.20	4.01	4.03	4.36	3.67	3.61
(e) a-GalNAc	99.6	51.6	67.9	77.5	72.4	60.7	
	5.00	4.25	4.12	4.09	4.35	3.67	3.61
(f) a-GalNAc	99.9	50.7	77.5	75.8	72.5	60.5	
	4.98	4.50	4.14	4.32	4.42	3.61	3.53
(g) ß-Glc	106.1	74.1	76.9	71.1	77.2	62.1	
	4.51	3.28	3.46	3.35	3.39	3.89	3.68

ABSTRACT OF THE DISCLOSURE

Mass spectrometry investigations of partially purified *C. jejuni* protein PEB3 showed it to be partially modified with an Asn-linked glycan of mass 1406 Da and comprised of one hexose, five HexNAc and a species of mass 228 Da, consistent with a trideoxydiacetamidohexose. By means of soybean lectin affinity chromatography, a mixture of glycoproteins was obtained from a glycine extract, and 2D-gel proteomics analysis led to the identification of at least 22 glycoproteins, predominantly annotated as periplasmic proteins. Glycopeptides were prepared from the glycoprotein mixture by pronase digestion and gel-filtration. The structure of the glycan was determined by using nano-NMR techniques to be GalNAc-α1,4-[Glcβ1,3-]GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac-β1,N-Asn-Xaa, where Bac is bacillosamine, 2,4-diacetamido-2,4,6-trideoxyglucopyranose. Protein glycosylation was abolished when the *pglB* gene was mutated, providing further evidence that the enzyme encoded by this gene is responsible for formation of the glycopeptide N-linkage. Comparison of the *pgl* locus with that of *Neisseria meningitidis* suggested that most of the homologous genes are probably involved in the biosynthesis of bacillosamine.

Figure 1

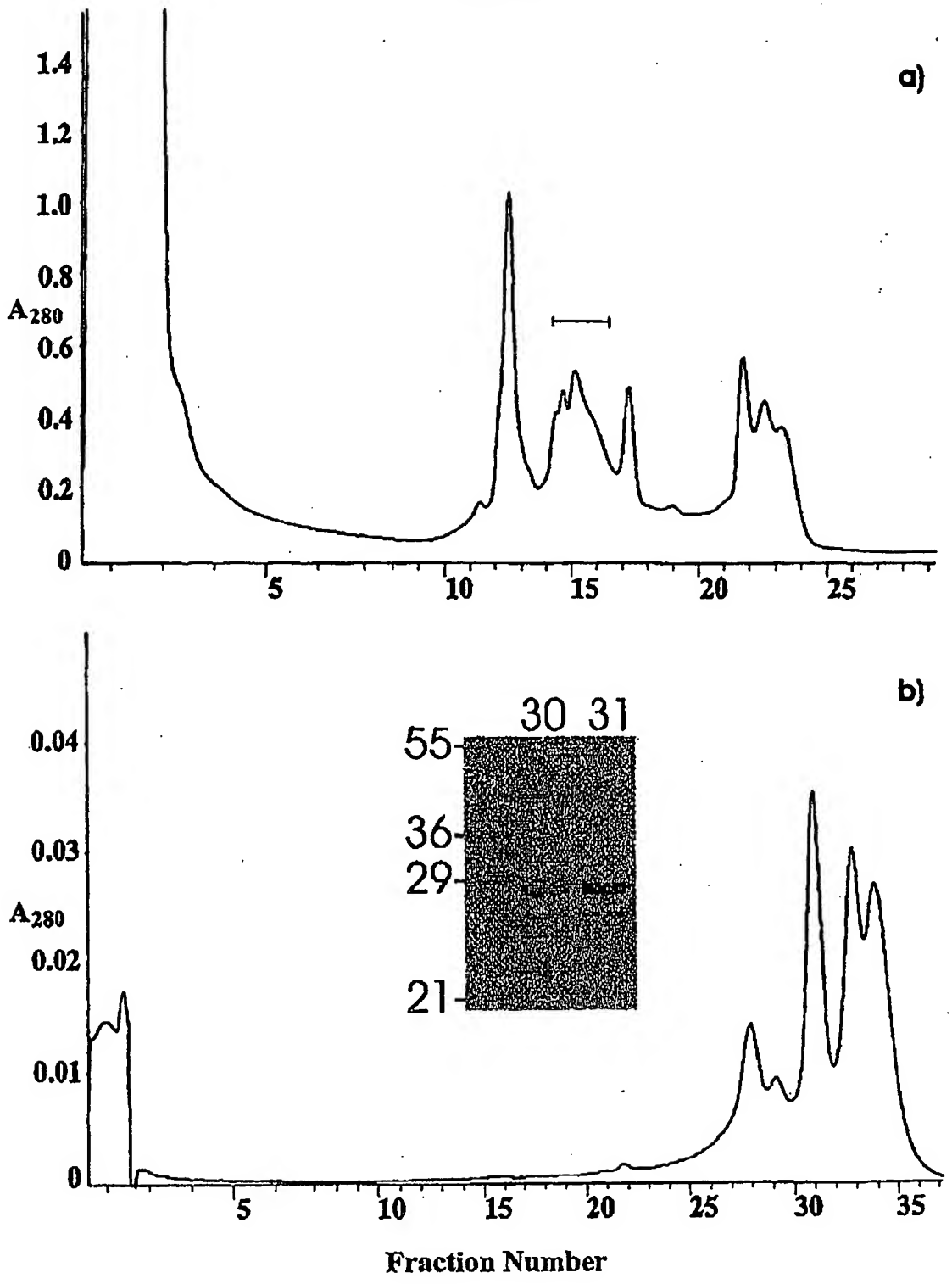


Figure2

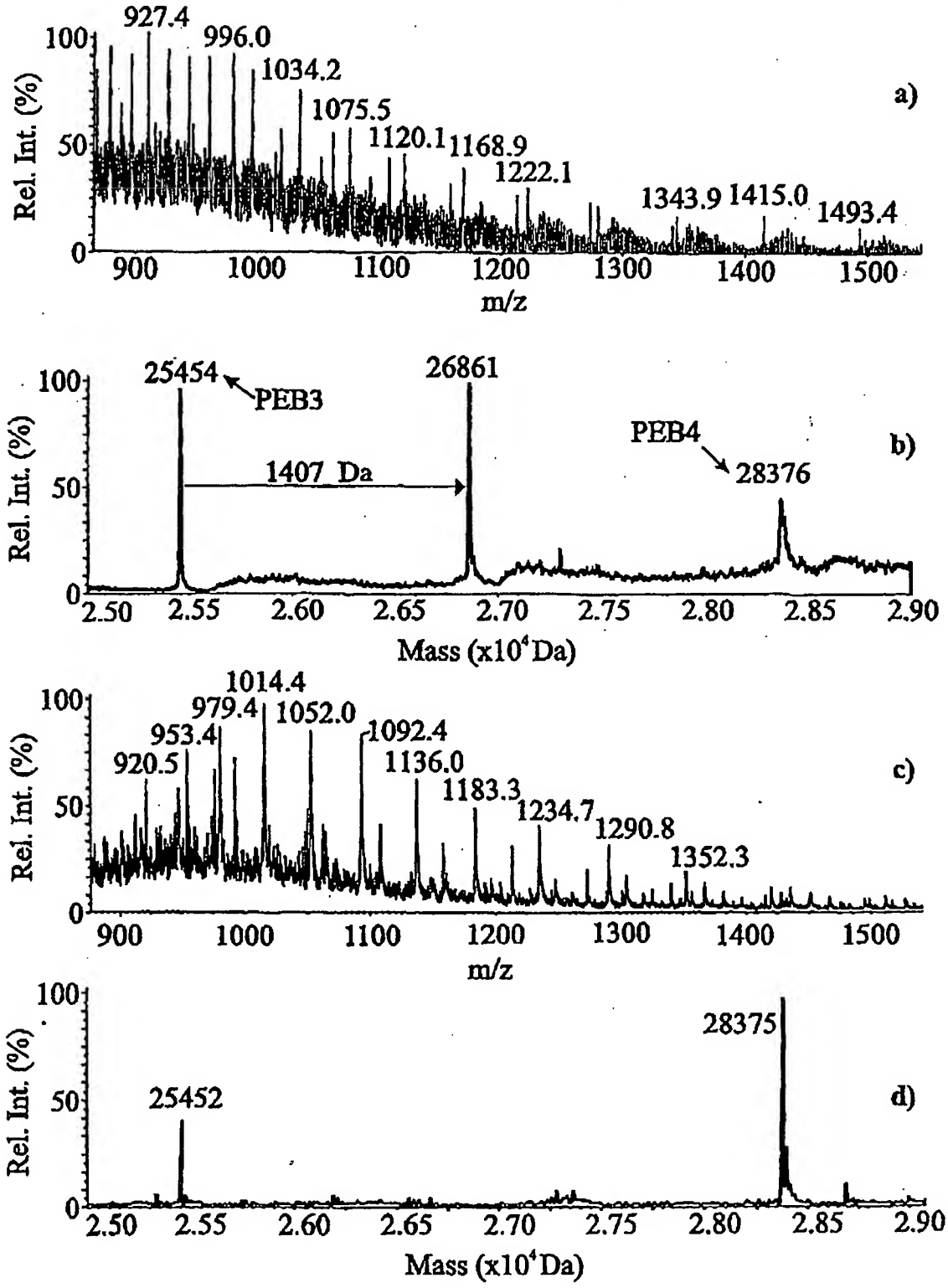

927.4
996.0
1034.2
1075.5
1120.1
1168.9
1222.1
1343.9
1415.0
1493.4
100
50
0
Rel. Int. (%)
900
1000
1100
1200
1300
1400
1500
m/z
a)
25454
PEB3
26861
PEB4
28376
1407 Da
b)
2.50
2.55
2.60
2.65
2.70
2.75
2.80
2.85
2.90
Mass (x10⁴Da)
1014.4
979.4
1052.0
953.4
1092.4
920.5
1136.0
1183.3
1234.7
1290.8
1352.3
c)
28375
25452
d)

Figure 3

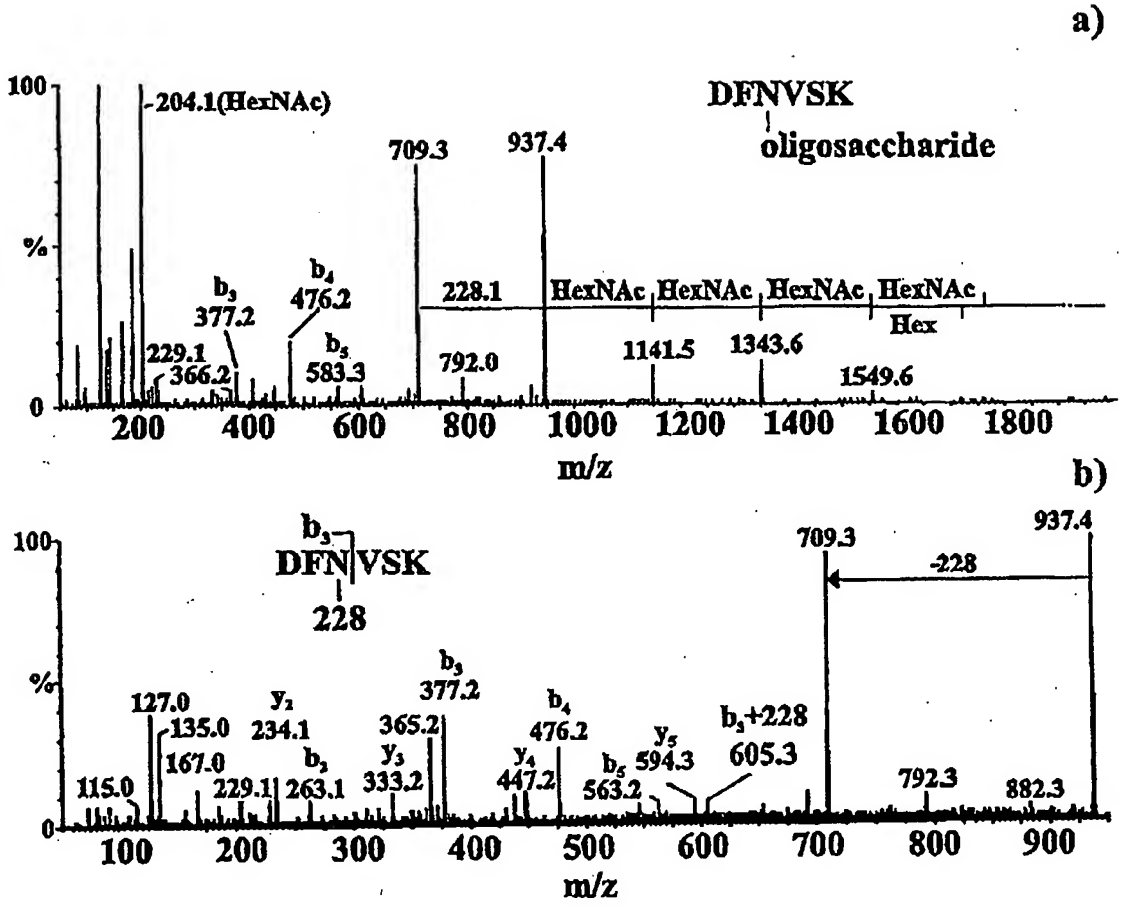

a)
100
%
0
-204.1(HexNAc)
709.3
937.4
DFNVSK
oligosaccharide
228.1
HexNAc
HexNAc
HexNAc
HexNAc
Hex
b3
377.2
b4
476.2
229.1
366.2
b5
583.3
792.0
1141.5
1343.6
1549.6
200
400
600
800
1000
1200
1400
1600
1800
m/z
b)
100
%
0
b3
DFNVSK
228
709.3
937.4
-228
b3
377.2
127.0
y2
234.1
135.0
167.0
b2
263.1
365.2
y3
333.2
b4
476.2
y4
447.2
b5
563.2
y5
594.3
b5+228
605.3
115.0
229.1
792.3
882.3
100
200
300
400
500
600
700
800
900
m/z

Figure 4

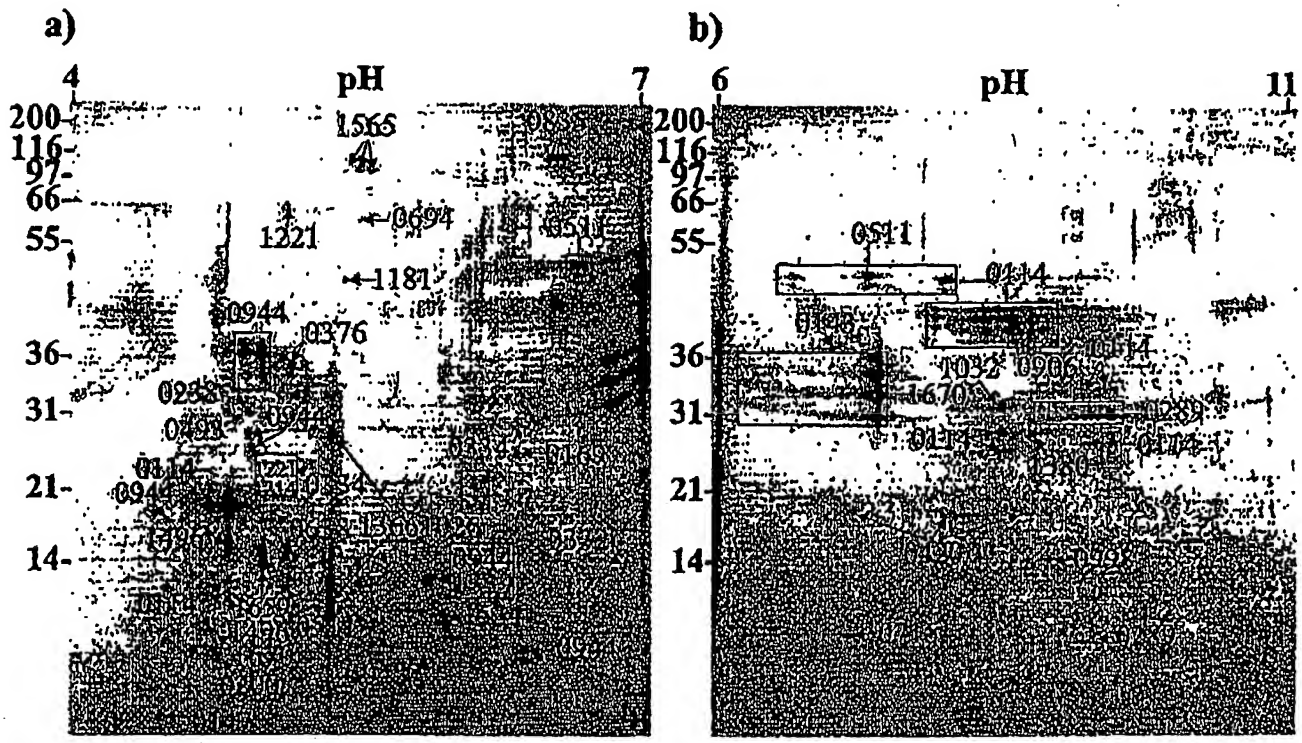


Figure 5

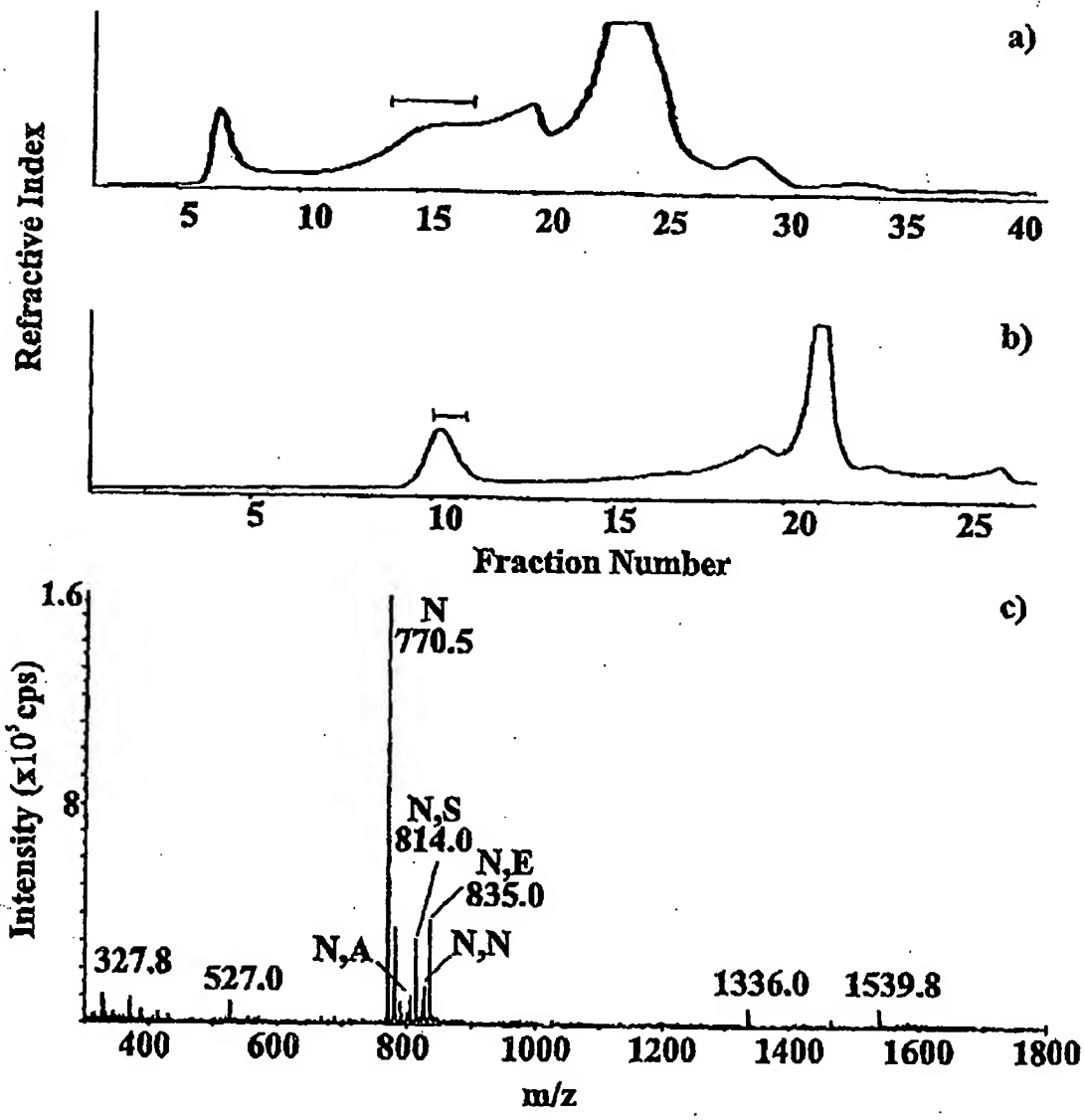

a)
b)
Refractive Index
5
10
15
20
25
30
35
40
Fraction Number
c)
1.6
8
Intensity (x10³ cps)
N
770.5
N,S
814.0
N,E
835.0
N,A
N,N
327.8
527.0
1336.0
1539.8
400
600
800
1000
1200
1400
1600
1800
m/z

Figure 6

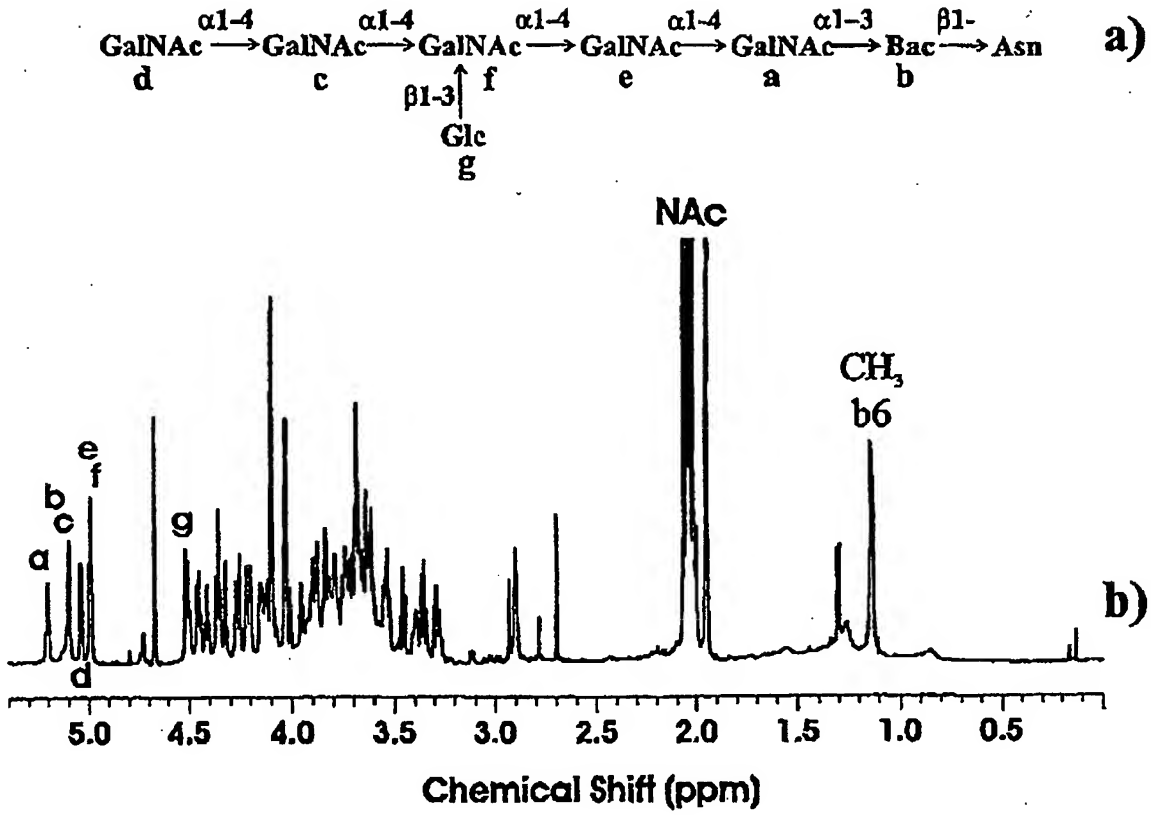

α1-4
α1-4
α1-4
α1-4
α1-3
β1-
GalNAc → GalNAc → GalNAc → GalNAc → GalNAc → Bac → Asn
d c f e a b
β1-3
Glc
g
a)
NAc
CH3
b6
e
f
b
c
a
g
d
b)
5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
Chemical Shift (ppm)

Figure 7

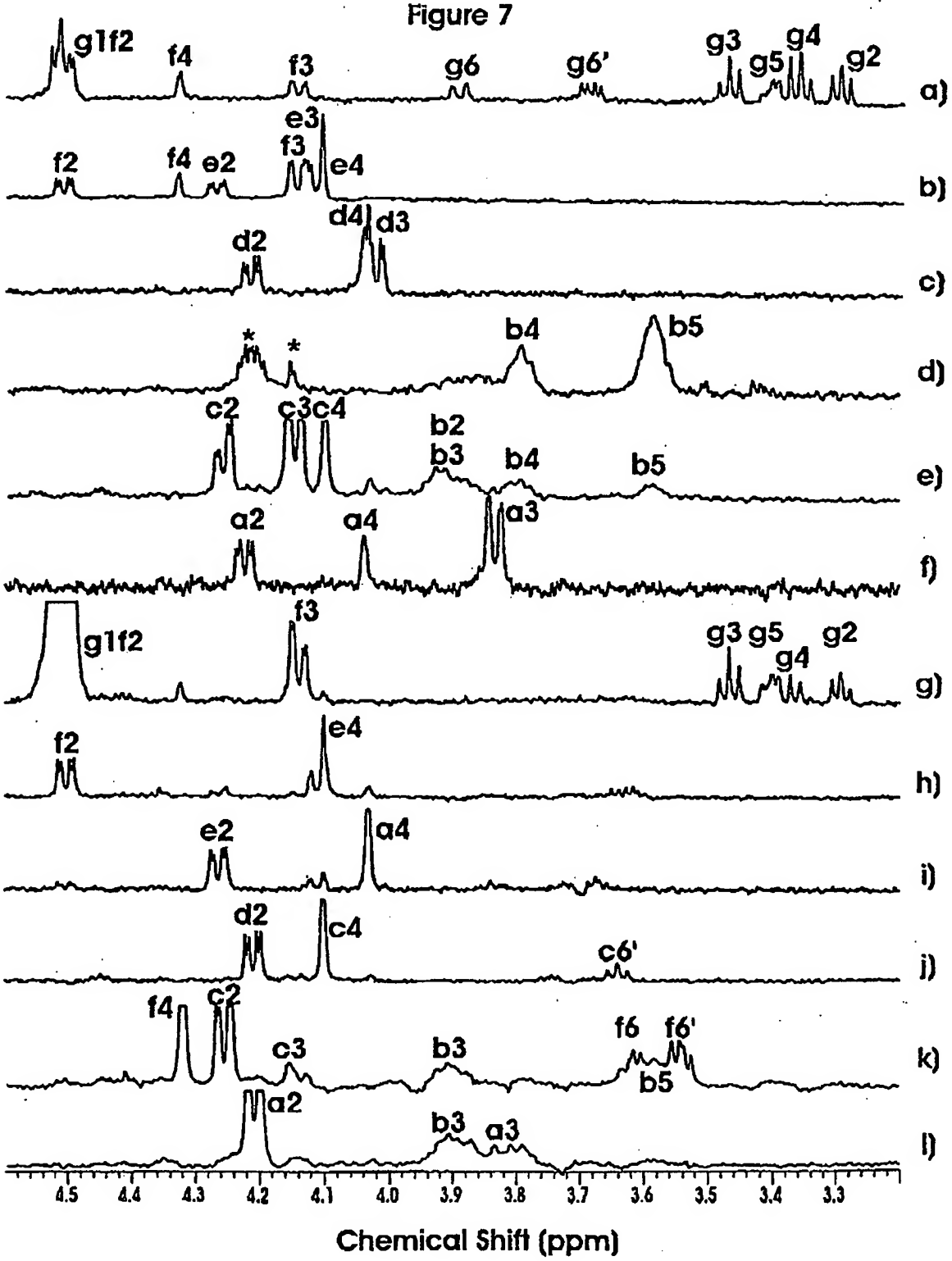
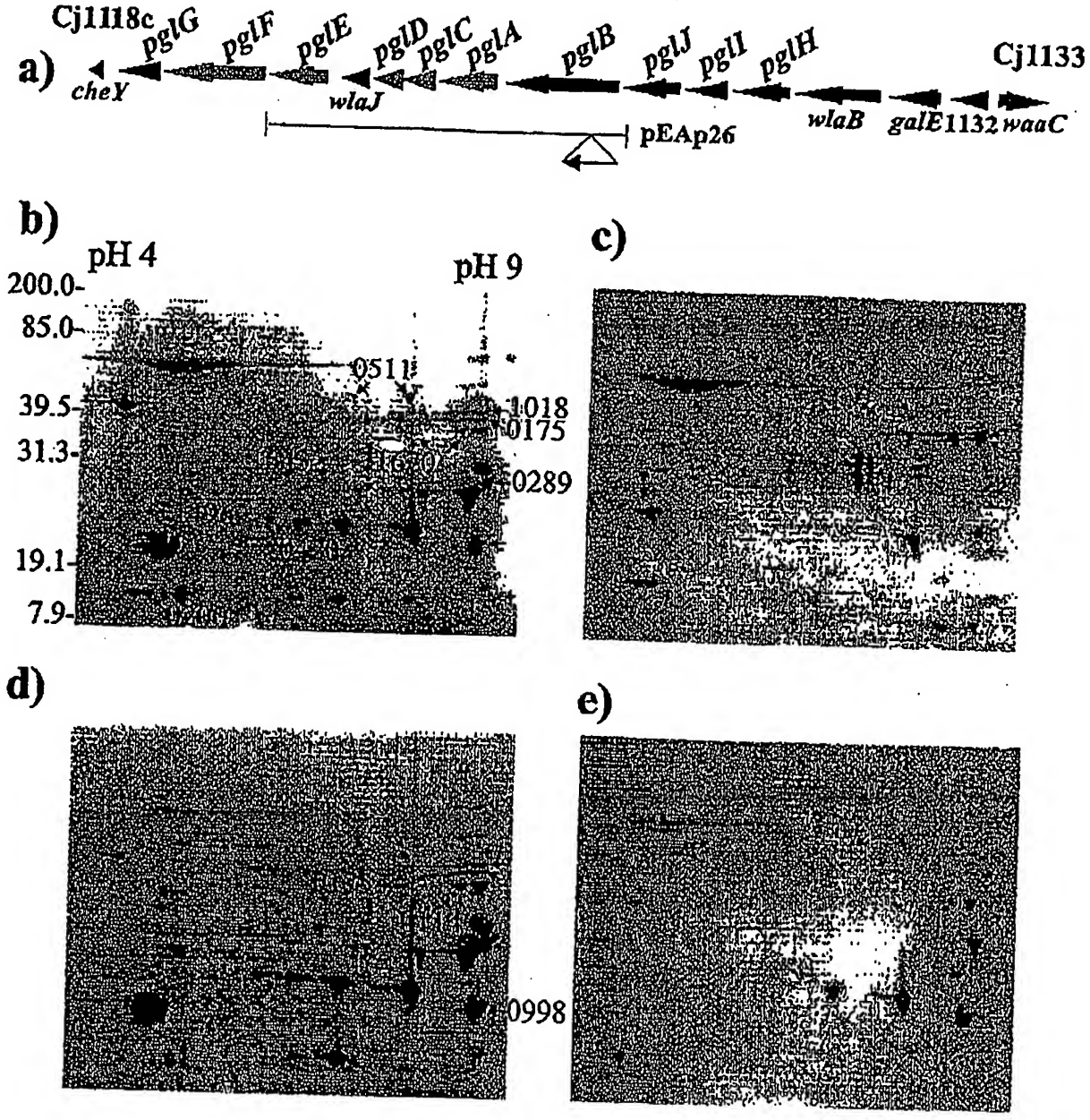

g1f2
f4
f3
g6
g6'
g3
g5
g4
g2
a)
f2
f4
e2
e3
f3
e4
b)
d4
d3
d2
c)
*
*
b4
b5
d)
c2
c3
c4
b2
b3
b4
b5
e)
a2
a4
a3
f)
g1f2
f3
g3
g5
g4
g2
g)
f2
e4
h)
e2
a4
i)
d2
c4
c6'
j)
f4
c2
c3
b3
f6
f6'
b5
k)
a2
b3
a3
l)
4.5
4.4
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
Chemical Shift (ppm)

Figure 8


a)
Cj1118c
pglG
pglF
pglE
pglD
pglC
pglA
pglB
pglJ
pglI
pglH
Cj1133
cheY
wlaJ
pEAp26
wlaB
galE1132
waaC
b)
pH 4
pH 9
200.0
85.0
39.5
31.3
19.1
7.9
0511
1018
0175
0289
c)
d)
0998
e)